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# Dietary Sulfate and the Excretion of Glycocholic and Taurocholic Acids in the Rat

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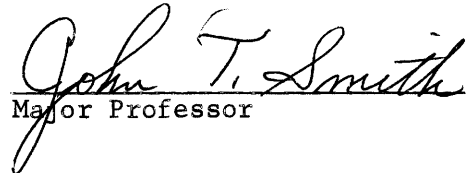
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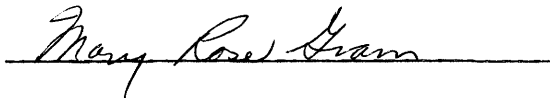
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Vice Chancellor for  
Graduate Studies and Research

DIETARY SULFATE AND THE EXCRETION OF GLYCOCHOLIC  
AND TAUROCHOLIC ACIDS IN THE RAT

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A Thesis

Presented to  
the Graduate Council of  
The University of Tennessee

---

In Partial Fulfillment  
of the Requirements for the Degree  
Master of Science

---

by  
Elfrieda Gayle Fuqua

August 1971

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## ABSTRACT

The relationship of dietary sulfate and the availability of taurine in the tissues to the relative conjugation of cholic acid with glycine and taurine to form the bile acids, glycocholic and taurocholic acids, in rats was investigated by feeding diets which contained different levels of total sulfur as sulfate and different neutral to inorganic sulfur ratios. Following stomach-tube feeding of cholic acid-24-<sup>14</sup>C, the relative conjugation, glycocholic:taurocholic ratio (G:T ratio), was determined by extracting the bile acids from the intestinal contents of the jejunum-ileum section of the small intestine. The bile salts were separated by thin-layer chromatography and visualized by an ethanolic phosphomolybdic spray. The appropriate spots were removed from the plates and the radioactivity of the samples evaluated by liquid scintillation.

The G:T ratio of animals fed the "normal" diet which contained 0.10 percent inorganic sulfur and 0.57 percent organic sulfur was the smallest indicating the highest relative conjugation with taurine. Increased taurine conjugation in relation to glycine conjugation has been associated with low serum cholesterol levels and limited atherosclerosis. The diet containing a high inorganic sulfur level (0.42 percent) and no added cysteine had the largest G:T ratio corresponding to the smallest relative amount of taurocholic acid. Likewise, in the diets which contained low levels of inorganic sulfur, the G:T ratios were larger than that of the "normal" diet. These data have been interpreted to indicate that either too high or too low a level of inorganic

sulfur causes changes in the relative conjugation of taurine. Requirements other than taurine availability which affect bile acid conjugation were also considered in relation to the present findings. The results of this study have been interpreted to imply that the level of inorganic sulfur in the diet may serve an important regulatory function in bile acid conjugation.

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## CHAPTER I

### INTRODUCTION

Recent evidence has indicated that feeding rats varying levels of inorganic sulfur caused different percentages of cysteine sulfur to be excreted as urinary taurine. It was demonstrated in the laboratories of the Department of Nutrition, The University of Tennessee, Knoxville, that rats fed diets containing low levels of inorganic sulfur excreted 50 percent more cysteine sulfur as taurine than did animals fed high levels of inorganic sulfur.

Bile acids which are synthesized by the demethylation and oxidation of cholesterol are conjugated with glycine and/or taurine before excretion into the bile. Conjugation of bile acids has been associated with cholesterol metabolism. Generally, taurine conjugation has been associated with low serum cholesterol and little atherosclerosis; whereas conjugation with glycine is associated with high serum cholesterol and increased evidence of atherosclerosis. The relative conjugation of the bile acids differs in species depending upon the presence of specific acyl-transferring enzymes in the microsomal particles of the hepatic cells. In rats, conjugation is primarily with taurine; whereas in man, more bile acids are conjugated with glycine. However, in many species particularly rat and man, the relative conjugation of cholic acid with glycine and taurine, i.e., the glycocholic:taurocholic ratio (G:T ratio) can be altered by dietary treatments which change the apparent availability of taurine.

Therefore, considering the inverse relationship between urinary taurine and dietary sulfate, and taurine conjugation and serum cholesterol, it seemed imperative to investigate the effect of inorganic sulfur on the G:T ratio in the small intestine of the rat. The investigation reported in this thesis was designed to study the effects of varying levels of dietary sulfate on the G:T ratio in the small intestine of adult male rats.

## CHAPTER II

### REVIEW OF THE LITERATURE

#### Introduction

The principal bile acids are cholic acid (3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ -trihydroxy-cholanic acid), chenodeoxycholic acid (3 $\alpha$ , 7 $\alpha$ -dihydroxycholanic acid), and deoxycholic acid (3 $\alpha$ , 12 $\alpha$ -dihydroxycholanic acid (1-3). Bile acids are the end products of the catabolism of cholesterol (4). Eighty percent of the total breakdown of cholesterol is eliminated via biliary excretion of bile acids (3). In addition, bile acids have many other physiological functions such as a role in fat absorption, cholesterol and steroid hormone absorption, a feed-back regulatory role in cholesterol synthesis as well as their own synthesis (5).

#### Biosynthesis of Bile Acids

Early research by Bloch et al. (6) and Zabin et al. (7) investigated the conversion of cholesterol to cholic acid. The bile acid molecule like that of cholesterol is a substituted cyclopentanoperhydrophenanthrene nucleus. It differs from cholesterol in that the isopropyl group (C-25, 26, and 27) is removed from the cholesterol side chain and the terminal carbon (C-24) is oxidized to form a carboxyl group. Also, the nucleus of the molecule is saturated so that the A ring is in cis configuration with the B ring; in addition, the C-7 and C-12 hydrogens of the cholesterol molecule are replaced by hydroxyl groups forming either dihydroxy or trihydroxy acids (5). A detailed review of the biosynthesis of cholic acid is discussed and illustrated by Haslewood (8).

Ayaki and Yamasaki (9) demonstrated that the biosynthesis of chenodeoxycholic acid occurs differently than does the biosynthesis of cholic acid and proposed a metabolic sequence in which there is a cleavage of the side chain of  $7\alpha$ -hydroxycholesterol before the transformation of the nuclear part of the compound thus forming  $3\beta$ ,  $7\alpha$ -dihydroxycholesterol-5-enoic as an intermediary compound. Then this unsaturated bile acid is converted to chenodeoxycholic acid which can be further oxidized to  $\alpha$ -muricholic acid, a trihydroxy bile acid.

Deoxycholic acid does not appear to be a normal metabolite of mammals. Rather it is the result of bacterial action on cholic acid (1).

#### Control of the Biosynthesis of Bile Acids

The biosynthesis of bile acids occurs in the liver of the rat and is regulated primarily by the amount of taurocholate reaching the liver by enterohepatic circulation (4). Shefer et al. (10) demonstrated that if sodium taurocholate was infused intraduodenally in adult male rats at concentrations greater than 10 mg per 100 g of body weight per hour, the biosynthesis of the primary bile salts was inhibited. Calculations from their data indicated that approximately 7.5 mg of taurocholate per 100 g of body weight per hour is needed to reach the liver in order to stimulate enterohepatic circulation in an intact animal. The rate of circulation of the taurocholate pool was 10-13 times per day; the pool size averaged 15 mg per 100 g rat. Shefer et al. (10) showed that to inhibit hepatic bile acid synthesis, at least 240 mg per 100 g rat per day must be administered. Even though the inhibitory effects of only taurocholate were shown in this study, the investigators suggested that

other bile salts and mixtures of bile salts might cause similar inhibiting effects.

Much research has been done in an effort to identify the sites of biosynthetic control of bile acid formation. The greatest amount of evidence presently seems to indicate that the  $7\alpha$ -hydroxylation of cholesterol to cholest-5-ene- $3\beta$ ,  $7\alpha$ -diol is the main rate-determining step (10-12). This particular step has been shown to be greatly influenced by biliary drainage. Complete drainage of the bile of the rat causes an increase of 10 to 15 times the synthesis measured in normal rats (11). Shefer et al. (12) also demonstrated that the administration of cholestyramine or phenobarbital also enhanced the hydroxylation. Cholestyramine probably functioned similarly as would biliary drainage due to its sequestering ability. Huff et al. (13) found that cholestyramine also increased the fecal bile acid excretion without effect on either the plasma or the liver cholesterol levels. Phenobarbital probably acts to stimulate the formation of endoplasmic reticulum whereby more microsomal enzymes are available to the liver (12).

More recent research by Shefer et al. (4) confirmed earlier observations of bile acid synthesis and suggested that a negative feedback control of the  $7\alpha$ -hydroxylase by cholesterol required an adequate supply of liver cholesterol for bile acid synthesis. Therefore, if cholesterol synthesis is inhibited, there would be a reduction in bile acid synthesis. Consideration of the evidence (14) which designates  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA reductase (HMG-CoA reductase)(E.C.1.1.1.34) as the rate-controlling enzyme in cholesterol biosynthesis makes it

reasonable to assume that any factors which affect HMG-CoA reductase would cause changes in bile acid synthesis as well as cholesterol synthesis. Shefer et al. (4) point out that there are other steps following the hydroxylation of cholesterol which could be controlling reactions in the synthesis of bile acids but conclude that these are of no major importance. Also, after this major controlling step and formation of 7 $\alpha$ -hydroxycholesterol, the quantity of bile acids produced is no longer controlled by the circulating bile acid pool.

#### Presence of Taurine in the Tissues

Just as the knowledge of the importance of various other sulfur-containing compounds is somewhat incomplete, the importance of taurine is also not fully understood. Since 1827, it has been known that taurine is a constituent of living organisms occurring in higher concentrations in animals than in plants. Taurine was first isolated from ox bile (15). Jacobsen and Smith (15) review in detail the taurine concentration in various animals as well as the various organs in the body of the animal.

Jacobsen and Smith (15) cited 1960 research by Schram which found that in the rat the taurine concentration was approximately 0.15 percent of the total body weight or 1,200  $\mu$ moles per 100 g of body weight. However, Boquet and Fromageot (16) found only  $663 \pm 60$   $\mu$ moles per 100 g of body weight in their investigations. The heart and the striated muscles contain approximately 75 percent of the total body taurine (17) with the spleen, bone marrow, and the thymus containing smaller amounts.

Taurine concentration in tissues is maintained at a somewhat constant level. Several factors have been identified which affect to

some extent the tissue levels as well as the excretion of taurine. Livers of female rats have about twice as much taurine as do livers of male rats (18-20); however, lung tissue in males has a higher taurine content than does female lung (21). Brain (22, 23), heart (19, 20), and muscle (19, 20, 22) contain similar amounts of taurine in both sexes. Age causes a decrease in taurine content especially evident in brain tissue (21). In fasting, taurine levels increase in the liver and muscle of male rats but decrease in the livers of female rats. Levels in kidney, heart, spleen, testis, and thymus are not affected (20).

Pyridoxine deficiency causes a decrease in urinary taurine excretion thought to be caused by a decrease in the activity of cysteinesulfinate carboxylase (E.C.4.1.1.29) which requires pyridoxine in order to function (24). However, there is not a tissue level decrease in rat brain, liver, spleen, or muscle (15).

Hormonal changes may also affect taurine tissue level and excretion to some extent. Following adrenalectomy, there is a decrease in liver taurine in female rats; but the liver, kidney, muscle, and spleen of male rats are not affected (20). Ovariectomy causes the high taurine levels in the liver of the female to decrease to levels similar to those of male rats; however, the administration of estrogens to male rats does not cause an increase in tissue levels of taurine (20). The effects of other hormones are not clearly known (15).

The effects of several different drugs, irradiation, electroshock, evisceration, partial hepatectomy, dehydration, and administration of  $\beta$ -alanine and  $\beta$ -aminoisobutyric acid have been reviewed (15).



### Biosynthesis of Taurine

The biosynthesis of taurine has been shown to occur by several pathways. Jacobsen and Smith (15) have reviewed and illustrated five different metabolic pathways. By considering these pathways, it can be seen that they all are interrelated. The main biosynthetic route especially evident in mammalian species involves the action of a decarboxylase enzyme on cysteinesulfinic acid. Taurine biosynthesis along this pathway requires the oxidation of cysteine to cysteinesulfinic acid, the decarboxylation of cysteinesulfinic acid to hypotaurine, and the oxidation of hypotaurine to taurine. A second possible pathway which also utilizes a decarboxylase enzyme differs from the above mentioned pathway in that cysteinesulfinic acid is oxidized to cysteic acid and then cysteic acid is decarboxylated to taurine (15). Jacobsen *et al.* (25) and Awapara and Wingo (26) agree that cysteinesulfinic acid is decarboxylated more rapidly than cysteic acid and thus is a preferred substrate. Even though it is agreed that the decarboxylation of cysteinesulfinic acid to hypotaurine is the most significant pathway to taurine synthesis, the biochemistry of the conversion of hypotaurine to taurine is still rather unclear (15). The absence of the decarboxylase enzymes in certain tissues and the presence of high concentrations of taurine in the tissues have been interpreted as evidence for additional pathways for taurine synthesis in some species (15).

Methionine (27, 28), methionine sulfoxide (29), homocysteine (30), cysteine (31, 32), and cystine (33-35) have been shown to be precursors of hypotaurine and taurine. Early research by White (36) demonstrated

that the addition of cholic acid to a diet low in sulfur-containing amino acids produced a cysteine deficiency accompanied by a suppression in growth of rats. However, the reduction in growth was shown to be reversed by feeding cysteine, methionine, or cystine but not taurine. Awapara (37) found that feeding cholic acid and/or taurine had no effect on taurine concentration in liver, kidney, spleen, heart, and muscle. The investigator concluded that taurine is synthesized at a somewhat constant rate and is relatively independent of the amount of taurine present in the diet. Therefore, only limited amounts of dietary taurine were used for taurocholic acid formation; and likewise, dietary taurine would not function to relieve cysteine/cystine deficiency.

#### Taurine Excretion

Taurine turnover rates in the rat are somewhat constant at approximately 35  $\mu$ moles per 24 hours per 100 g of body weight. The half-life of total taurine is about 12 to 13 days in an adult but somewhat shorter in younger animals. It has been found by injecting  $^{35}\text{S}$ -taurine into rats, approximately 46 percent of the taurine in the body was excreted in the urine; less than 10 percent was eliminated in the feces; the remaining 44 percent stayed in the tissues (38). However, the excretion fluctuates according to several factors such as genetics, age, sex, diet, vitamins, and hormones. These factors have been reviewed by Jacobsen and Smith (15) and Whittle (39).

Another factor which affects taurine excretion and which is essential to this research is the level of inorganic sulfur in the diets fed to rats. Whittle (39) found that when rats were fed diets containing

similar levels of total sulfur but varying levels of inorganic sulfur as sulfate, more  $^{35}\text{S}$ -cysteine was excreted as  $^{35}\text{S}$ -taurine by animals fed diets containing the low levels of inorganic sulfur (0.0002 percent). Likewise, there was an increase in urinary taurine excretion from rats fed diets with low levels of inorganic sulfur when the diets were all supplemented with the same amounts of organic sulfur as cysteine. Thus, from these data, it would seem appropriate to propose that the glycocholic:taurocholic ratio (G:T ratio) in rats should be affected by the amount of inorganic sulfur in the diets.

#### Conjugation of Taurine

Although the metabolism of taurine is not clearly understood, certain physiological roles have been identified. One of the most important is its role in bile acid conjugation (15). As has been indicated earlier in this review, bile acids are synthesized in the breakdown of cholesterol; thus taurine might also be considered to play a role in the elimination of cholesterol from the body. The majority of the bile acids are conjugated with either glycine or taurine before excretion into the bile. Free bile acids are not found in bile under normal conditions. The enzyme system necessary for the conjugation is found only in the microsomal particles of the liver cell (40). Bremer (40) found that isolated microsomes catalyze bile acid conjugation when adenosinetriphosphate (ATP), coenzyme A, and magnesium ions are available. He also reported that conjugation involves the formation of cholyl-S-CoA as an intermediate and requires two different acyl-transferring enzymes which transfer the bile acyl group from the coenzyme A to taurine or

glycine. The ratio of glycine- and taurine-conjugated bile acids varies with mammalian species due largely to the activities of these two enzymes which catalyze the conjugation. Haslewood (8) and Bremer (40) both state that there was an obvious association between the type of bile acid conjugation and the kind of diets characteristic of the particular species. Carnivorous vertebrates primarily conjugate taurine; whereas herbivorous vertebrates form mainly glycine conjugates and omnivorous animals exhibit various proportions of both conjugates.

More specifically, Awapara (18) reported no detectable amount of taurine-conjugated bile acids in the rabbit, thus, seemingly, this indicates that the rabbit lacks the specific enzyme necessary for taurine conjugation. The pig also conjugates almost all the bile acids with glycine. In the rat, Bremer (41) found that taurine conjugation was more than twice as efficient as was glycine conjugation. The bird and dog also exhibit preference for taurine conjugation (42). The bile acids in man and other primates are conjugated in variable proportions between taurine and glycine. Sjövall (1) found that in man 65 to 75 percent of the total bile acids were glycine conjugates; however, after feeding taurine for 5 days, 96 percent of the bile acids were then taurine-conjugated. Thus, it seems that in man where enzymes for glycine as well as taurine conjugation are present, the G:T ratio may be greatly affected by the amount of taurine available. This same reasoning makes it logical to propose that the G:T ratio in rats could also be affected by the amount of taurine available.

### Absorption and Transport of Bile Acids

One of the crucial factors affecting the proper enterohepatic circulation of bile acids is their absorption from the gastrointestinal tract. Absorption of bile acids is greatly dependent upon their molecular structures and micellular formations. As pointed out earlier, the bile acid molecule closely resembles the cholesterol molecule with the exception of the side chain and the replacement of hydrogen with hydroxyl groups (5). Conjugation with glycine or taurine changes the  $pK_a$  from 6.0 for unconjugated acids to 4.0 for glycine conjugates and 2.0 for taurine conjugates. Also, bile acid molecules have the ability to form micelles when the concentration of the solution reaches the critical micellular concentration (CMC). Micellular formation is dependent upon such factors as temperature, pH, and the presence of counter-ions. All these factors affect the absorption of bile acids (5). Eriksson (43) estimated that the total bile salt pool varies from 20.3 to 34.4  $\mu$ moles. Using calculations of Dietschy et al. (44), this would suggest that the total bile salt pool could be absorbed every 49 to 90 minutes. Olivecrona and Sjövall (45) reported data which were interpreted to indicate that an amount of bile salt which corresponds to the small intestinal pool can be delivered to the liver via the portal vein once every 110 minutes.

Early research provided evidence that bile acids were most readily absorbed from the ileum; however, later evidence permitted the conclusion that absorption also occurred in the proximal small intestine (5). Most recent investigators (46-50) have suggested that the majority of bile acid

absorption takes place in the distal small intestine; however, some limited absorption occurs in the proximal section as well.

Dietschy et al. (44) found three transport mechanisms for bile acid absorption - active transport, passive ionic, and passive non-ionic diffusion. Later, Dietschy (5) reviewed micellar diffusion as a fourth absorption mechanism. Several investigators (51-53) have emphasized that bile acids are absorbed by active transport only in the ileum. Clearly, bile acids are found to be moving against an electrochemical gradient. Sites of active transport increase down the length of the ileum and are in most abundance at the terminal end of the small intestine (5). Both cholate and taurocholate are actively transported due to the lack of discrimination of the transport carrier (52). The rate of active transport of taurocholate equals 1.26  $\mu$ moles per minute per cm and 0.63  $\mu$ moles per minute per cm for cholate (44).

Since transport by passive diffusion occurs only with a favorable electrochemical gradient, the movement of bile acids is into the portal blood from the intestinal lumen (54). In passive ionic diffusion, the rate of diffusion depends upon the structure of the bile acid. The rate is inversely related to the number of hydroxyl groups and to the size of the moiety conjugated at the C-24 position (5).

Because the  $pK_a$  for unconjugated bile acids is higher than that of conjugated acids, unconjugated bile acids may exist in the un-ionized form at physiological pH's (5). Dietschy et al. (44) found that 61 percent of the unconjugated acids are absorbed by non-ionic diffusion at pH 6.2. Also, absorption of un-ionized bile acids was 5 to 6 times greater than

absorption of ionized forms. This mechanism of absorption could only occur in areas of the gastrointestinal tract where the bile acids are present in their deconjugated forms. Thus, according to Dietschy (55), non-ionic diffusion would occur in the distal ileum and colon.

Norman and Sjövall (56) have revealed that deconjugation of bile acids does not start until the bile salts have entered the cecum. The splitting of the pseudo-peptide bond between cholic acid and glycine or taurine is due primarily to the action of microorganisms present in the large intestine. Gustafsson et al. (57) showed that digestive enzymes had no effect on the breakdown of these acids. Normal or conventional rats were found to metabolize cholesterol faster than do germfree rats (58) and excrete in the feces 90 percent more bile acids which were completely deconjugated (59). Wostmann et al. (58) also found that the bile acids excreted in the feces of germfree rats consisted of  $\alpha$ -muricholic,  $\beta$ -muricholic, and cholic acid all conjugated with taurine.

#### Concentration and Excretion of Individual Bile Acids

The excretion and relative concentration of individual bile acids have been shown to be affected by several factors such as dietary cholesterol, tissue cholesterol, hormones, and diet. Beher et al. (60) in supplementing the diets of rats with 3 percent corn oil, 1 percent cholesterol alone and 1 percent cholesterol with 3 percent corn oil found that the total bile acid pool size was not greatly affected; however, with cholesterol feeding, there was a decrease in cholic acid and a comparable increase in chenodeoxycholic acid concentration. These same results were obtained when the diet was supplemented with 1 percent

cholesterol and 3 percent corn oil, thereby supporting the findings of Siperstein and Chaikoff (61) that unsaturated oils do not affect the rate of bile acid excretion in the rat. These differences in the relative concentrations of cholic and chenodeoxycholic acids in the pool are partially because the chenodeoxycholic acid turnover rate is faster than that of cholic acid. The excretion of bile acids is directly proportional to the half-life of the bile acid pool.

Somewhat similar results were obtained when tissue cholesterol levels were elevated by drug treatment, i.e., cholic acid. The total bile acid pool concentrations were constant with the same shifts in the relative concentrations of cholic and chenodeoxycholic acids as shown by dietary cholesterol (60). Beher et al. (60) suggest that since the half-lives of both bile acids decreased, there are probably differences in the rates of synthesis of the primary bile acid by the liver mitochondria. They also suggest that the half-lives are decreased due to decreases in transport across the walls of the small intestine and cecum. The bile acid excretion rate increased two-fold. Two reasons for this increased elimination were given. First, by increasing the bile acid turnover rate, the rate of conversion of cholesterol to bile acids is also increased due to a decrease in the negative feedback inhibition. Second, the spectrum of the bile acid pool moves toward the acids with higher turnover rates. Investigation of  $\beta$ -sterol concentrations in the small intestine and cecum indicated that in the presence of elevated tissue cholesterol there was no increase in the mobilization of cholesterol by the fecal sterol pathway.



The factors which cause the differences in the relative concentration of cholic and chenodeoxycholic acids are not completely understood. In the case cited above, Beher et al. (60) showed a difference in the relative concentration of cholic and chenodeoxycholic acids when cholesterol was fed to rats. Eriksson (62) found similar differences caused by the administration of thyroid hormone to rats. In hypothyroidism, less than 10 percent of the total bile acid output is chenodeoxycholic; whereas 70 percent of the total output is of chenodeoxycholic in hyperthyroidism as compared to 15 percent in the normal rat. The administration of D- and L-triiodothyronine in rats (63) causes the secretion pattern of the bile acids to be identical to that found in hyperthyroidism. The secretion of bile acids in hyperthyroid rats increased to twice that of the control group. Similarly, Hellström and Lindstedt (64) found increased chenodeoxycholic in relation to the cholic acid in humans in a hyperthyroid state; however, this observation is in contrast to the findings of Failey et al. (65) who found a decrease in chenodeoxycholic acid when thyroid hormone was administered. Propylthiouracil treated rats produced only about one-fifth the total bile acids found in normal or thyroid-treated rats and resulted in a decrease of chenodeoxycholic acid as exhibited by a reduction in the taurochenodeoxycholic:taurocholic ratio (63). Lindstedt et al. (66) found that liquid formula diets containing corn oil produced a lower chenodeoxycholic acid concentration in man than a solid diet containing coconut oil yet exhibited no consistent changes in size of bile acid pool.

### Importance of Inorganic Sulfur in the Diet

Early research seemed to indicate that inorganic sulfur in the diet of a monogastric animal served no important physiological functions; however, recent research has found this finding to be erroneous. Pendergrass (67) found that amino acid sulfur could be converted to sulfate if vitamin E was present; Michels and Smith (68) then concluded that this observation indicated that inorganic sulfur could provide some nutritional benefits. Button et al. (69) found that animals absorbed more inorganic sulfur when fed diets low in organic sulfur. Also, when inorganic sulfur was included in the diet, feed efficiencies were higher. These investigators suggested that by limiting the level of inorganic sulfur in the diet, there was a drain on the sulfur-containing amino acids. Brown et al. (70) studied the effect of feeding low levels of inorganic sulfur on collagen metabolism. Gilmore (71) found greater sulfur absorption when rats were fed diets low in inorganic sulfur and supplemented with methionine. Cysteine metabolism has also been shown to be related to the level of inorganic sulfur in the diet (72). As discussed previously, Whittle (39) found a relationship between level of inorganic sulfur and urinary taurine excretion. Therefore, from the above data, it can be seen that inorganic sulfur does function in the body and is able to fill some of the requirements demanded of organic sulfur.

### Role of Bile Acids in the Control of Cholesterol Biosynthesis

The precise role of bile acids in cholesterol synthesis has not been completely elucidated. According to current concepts, the liver and

the gastrointestinal tract are the two major sources of circulating serum cholesterol (73, 74). Dietschy (73) found that bile salts were responsible for inhibition of cholesterologenesis in the intestine. The sites of inhibition were in the intestinal crypts in the small intestine. The inhibition in the biochemical synthesis sequence was localized to the step catalyzed by HMG-CoA reductase. Thus, due to previous research, it was proposed that bile acids exert a direct inhibitory effect upon hepatic cholesterologenesis similar to that exhibited in the intestine (74). Previously, it has been reported that bile acids added to liver slices in vitro inhibited cholesterol synthesis (75); biliary diversion (76) and cholestyramine feeding (15) enhanced cholesterologenesis in the liver; and, in man, increasing the size of the bile acid pool decreased sterol synthesis; whereas interruption of enterohepatic circulation increased synthesis (77). However, other data have been used to question the hypothesis that bile acids have a direct inhibitory effect on cholesterol synthesis. When added to liver slices in vitro, pure conjugated bile acids do not inhibit sterol synthesis (78); whereas pure unconjugated acids do (55). Work by Weis and Dietschy (74) showed that biliary diversion and biliary obstruction cause increased sterol synthesis. This effect is not influenced by dietary habits as evidenced by an enhanced hepatic cholesterologenesis during fasting. Therefore, it seems likely that bile acids do not exert a direct regulatory effect on cholesterol synthesis. After further work, Weis and Dietschy (74) suggested that hepatic cholesterologenesis may be a result of changes in the enterolymphatic circulation of cholesterol. Diverting both biliary hepatic and

lymphatic hepatic circulation had no additive effect on cholesterogenesis; yet diversion of either enhanced the hepatic HMG-CoA reductase activity. The increased cholesterogenesis experienced after biliary diversion was not prevented by restoration of enterohepatic circulation of bile acids but by the restoration of enterohepatic cholesterol circulation. In total, hepatic cholesterogenesis is controlled by the amount of endogenous and exogenous cholesterol which reaches the liver through enterohepatic circulation (74) and by the negative feedback mechanism at the HMG-CoA reductase level (78, 79). So the role that bile acids play in hepatic cholesterogenesis is more specifically their action on the enterolymphatic circulation of cholesterol.

#### Relationship of Bile Acid Conjugation and Cholesterol Metabolism

The conjugation pattern of bile acids is also related to cholesterol metabolism and in some cases the concurrent development of atherosclerosis. Generally, taurine conjugation is associated with low serum cholesterol and a slight tendency toward atherosclerosis; whereas glycine-conjugated bile acids are associated with a high serum cholesterol level and more tendency toward atherosclerosis. The dog and rat which primarily conjugate bile acids with taurine have been found to be resistant to hypercholesterolemia; whereas man and rabbit, two species that conjugate the majority of their bile acids with glycine, are very susceptible to hypercholesterolemia and atherosclerosis (41).

Two possible mechanisms have been considered to determine the relative conjugation of bile acids. One mechanism emphasizes that the G:T ratio is determined by the amount of unconjugated bile acid which

must be processed by the liver. Thus, the rate of synthesis of primary bile acids, the amount of unconjugated bile acids returning to the liver by enterohepatic circulation, and the availability of glycine and taurine to hepatic cells would all affect the relative conjugation of bile acids (80). The second mechanism emphasizes events which deal with intestinal absorption. Preferential absorption of glycocholic or taurocholic acid (81) and greater affinity for bacterial enzymatic deconjugation of one conjugated form could alter the relative conjugation of excreted bile acids (80).

The relative bile acid conjugation has been shown to change throughout the life cycle of man. The average G:T ratio in adult man is approximately 3:1 with a range of 1:1 to 5:1. In a one-month fetus and infants up to approximately 10 days after birth, taurine-conjugated bile acids predominate; thereafter, glycine conjugation increases until at an age between 7-12 months, the ratio stabilizes at a level comparable to adult values. The reason for this sequence is not completely understood; however, it has been found that serum taurine levels are higher in infants than in adults (15).

The conjugation of bile acids can be manipulated by changes in diet. Oral administration of a taurine load in a normal adult causes increased taurine conjugation; however, in glycine feeding, no such changes are seen (1). Cysteine loading has a similar effect as taurine feeding except not to as great an extent (80). As was pointed out earlier, liquid formula feeding and changes in dietary fat composition by coconut oil substitution cause a decrease in the G:T ratio (60).

To help in understanding mechanisms responsible for the relative conjugation of bile acids, more information is needed on the factors which govern the availability of taurine for conjugation.

## CHAPTER III

### EXPERIMENTAL PROCEDURES AND METHODS

The effects of varying levels of dietary sulfate on the relative conjugation of bile acids with glycine and taurine by rats were investigated in an attempt to observe any relationship between the conjugation of taurine, its availability in the tissues of animals, and the influence of diets which contained different levels of total sulfur as sulfate and different neutral to inorganic sulfur ratios. These diets were composed of the basal diet shown in Table 1 and supplemented to the dietary composition indicated in Table 2. These diets were the same as those used by Whittle (39). Three lots of the basal diet were supplemented with calcium sulfate and cysteine to produce diets with inorganic sulfur levels of 0.0002 percent (Diet 1), 0.10 percent (Diet 3), and 0.42 percent (Diet 4) while maintaining the total sulfur as sulfate at the 0.67 percent level. In these diets as the inorganic sulfur increased cysteine decreased. In order to keep cysteine constant, two additional diets were prepared to which cysteine was added at the same level as it was added in the 0.10 percent inorganic sulfur diet, 0.40 percent. This gave one diet (Diet 2) with a total sulfur as sulfate content of 0.57 percent and an additional diet (Diet 5) with a total sulfur of 0.99 percent.

Calcium carbonate was added to maintain the calcium content constant; the non-nutritive bulk was added to adjust the weight. The salt mixture was a low-sulfate adaptation of the Hubbell et al. salt mixture (82).

TABLE 1  
Composition of basal diet

Component	g/100 g diet
Casein	15.00
Sucrose	30.00
Cornstarch	30.00
Cod-liver oil	2.00
Vegetable shortening <sup>a</sup>	6.00
Vitamin mixture <sup>b</sup>	2.00
Basal salt mixture <sup>c</sup>	1.34

<sup>a</sup>Crisco, Procter and Gamble, Cincinnati, Ohio.

<sup>b</sup>Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

<sup>c</sup>Source: Pendergrass, B. J. 1961 The interrelationship of tocopherol and sulfur metabolism. Unpublished Master's Thesis, The University of Tennessee, Knoxville.



TABLE 2  
Variations of basal diet

Diet	Additions to Basal Diet				Levels of Dietary Sulfate		
	CaSO <sub>4</sub> ·2H <sub>2</sub> O	CaCO <sub>3</sub>	Cysteine	Non-nutritive Bulk <sup>a</sup>	Inorganic SO <sub>4</sub>	Organic S as SO <sub>4</sub>	Total S as SO <sub>4</sub>
	g/100 g diet	g/100 g diet	g/100 g diet	g/100 g diet	g	g	g
1	0	1.34	0.53	11.79	0.0002	0.67	0.67
2	0	1.34	0.40	11.92	0.0002	0.57	0.57
3	0.18	1.23	0.40	11.85	0.10	0.57	0.67
4	0.75	0.91	0	12.00	0.42	0.25	0.67
5	0.75	0.91	0.40	11.60	0.42	0.57	0.99

<sup>a</sup> Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

Five groups of randomly selected male albino rats of the Sprague-Dawley strain weighing between 200-250 g were used for this study. These animals were obtained as weanlings and fed Purina Laboratory chow. Each group of eight animals was fed a different experimental diet. Distilled water and experimental diets were fed ad libitum for 7 days. The results of a previous investigation (39) indicated that an adaptation to the low sulfate diet might occur if the feeding period was extended for more than 7-14 days. A more recent investigation<sup>1</sup> has shown that the maximum effect of feeding a diet low in sulfate could be observed in 7 days. Therefore, at the conclusion of a 7-day experimental feeding period, each animal was administered by stomach tube a test dose of 0.5 ml <sup>14</sup>C-carboxy sodium cholate which contained 50 mg of <sup>14</sup>C-carboxy sodium cholate with a specific activity of  $8.4 \times 10^5$  counts/min/mg (cpm) and returned to the ad libitum feed and water regimen described above. Preliminary results collected in the laboratory of the Nutrition Department, The University of Tennessee, Knoxville, indicated the highest degree of specific activity in the contents of the small intestine was found 24 hours after administration of the <sup>14</sup>C-carboxy sodium cholate. Also, Norman and Sjövall (56) found no difference in the distribution of radioactive isotope in 24 hours and 70 hours. Thus, exactly 24 hours after the administration of the radioactive test dose, the rats were decapitated. The section between the jejunum and ileum of the small intestine was removed and stored at -20°C until analysis. Norman and Sjövall (56) found that conjugated bile

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<sup>1</sup>Smith, J. T. Unpublished observations.

acids were primarily confined to the small intestine. Less than 20 percent of the bile acids in the cecum were found to be conjugated; even smaller amounts were found in the contents of the lower large intestine. Microorganisms present in the large intestine cause deconjugation by splitting the pseudo-peptide bonds of the bile acids.

The bile acids were extracted from each intestine by the methods of Norman and Sjövall (56). Each intestine was homogenized in an Eberbach 8580 Semi-micro stainless steel blender with 40 ml of 80 percent ethanol. After homogenization, the mixture was poured into 100-ml Erlenmeyer flasks. The blender was rinsed with 20 ml of 80 percent ethanol and the rinsings were poured into the flasks. The bile acids were extracted by refluxing for 2 hours in a 100°C water bath. The samples were then centrifuged for 10 minutes at 1000 x g. The supernatant fluid was collected and evaporated to dryness in a convection oven.

The bile acids were separated by thin-layer chromatography by modifications of the methods of Anthony and Beher (83). Plates were made with acidic Silica AR-4G. Anthony and Beher (83) reported that the acidification of the adsorbent to pH 3.0 improved the sharpness of the bile acid spots. The Camag applicator was used to make 0.3 mm thick plates. They were dried for 5 minutes at room temperature to allow the adsorbent to set and then were activated for 2 hours in a 110°C drying oven. As recommended by Anthony and Beher (83), linear grooves or channels were marked on plates to delineate an adsorbent column and prevent a concave solvent front.

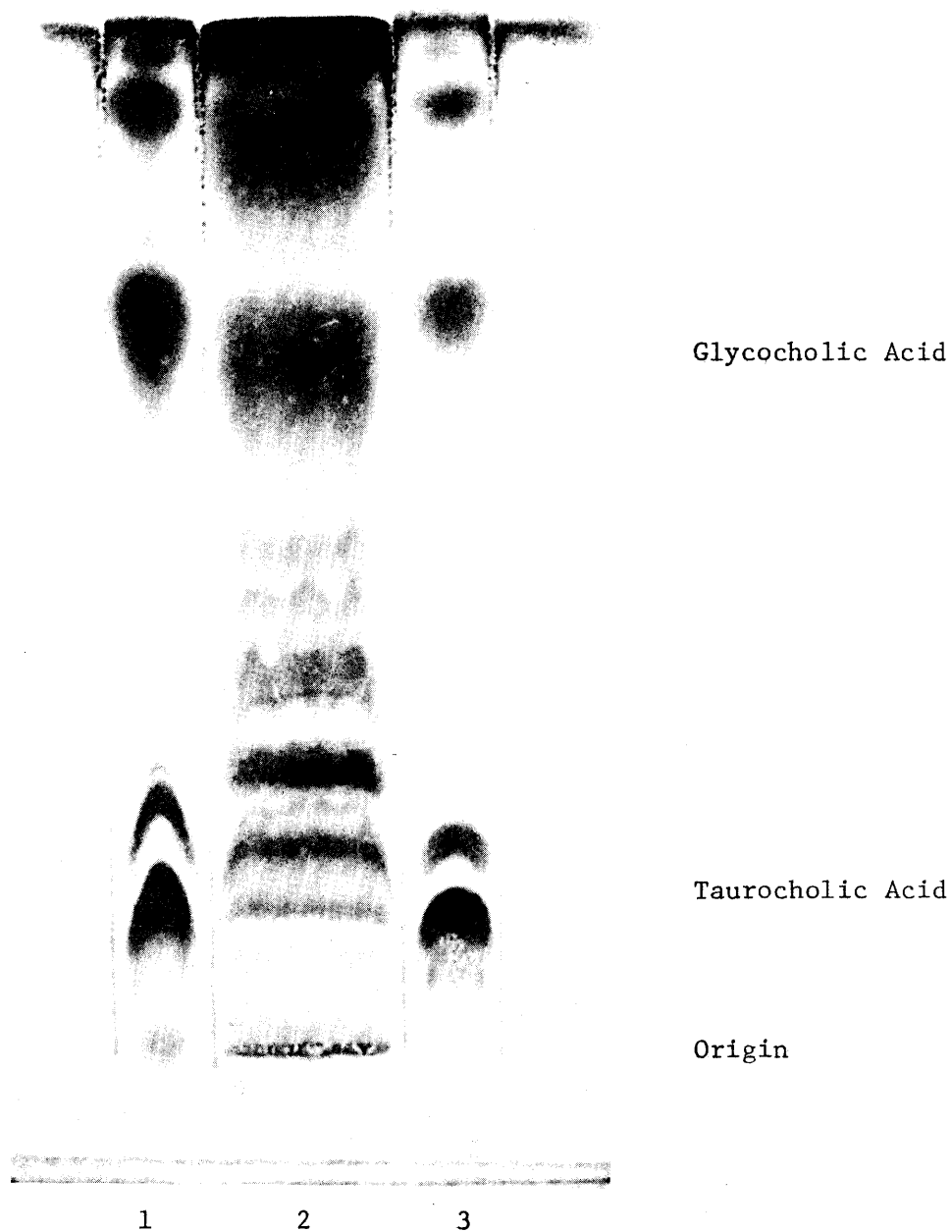
After reconstitution of the dried sample with 1 ml of 80 percent

ethanol, 30  $\mu$ l were stripped 1.7 cm from the bottom of the center adsorbent column. The column on the left was spotted with 20  $\mu$ l of the glycocholic standard and the right column with 20  $\mu$ l of the taurocholic standard. These standards were prepared by mixing 300 mg of each standard in 20 ml of 80 percent ethanol. After the samples were applied to the plates, they were then dried in a Freas vacuum oven for 15 minutes at 30 inches of mercury at 38°C to insure complete dryness.

The solvent system used was Solvent II by Hofmann (84), iso-amyl acetate:propionic acid:propanol:water (20:15:10:5), for the separation of conjugated bile acids. The plates were allowed to develop in equilibrated chambers at room temperature for 1 to 3 hours depending upon the ambient conditions. Plates were removed before the solvent front reached the top of the plate.

The plates were developed twice by an ascending technique and were dried in a vacuum oven for 15 minutes between developments. After the second development, the plates were thoroughly dried at 110°C for 3 hours. After cooling, the bile acid spots were visualized by spraying the plates with freshly prepared 10 percent phosphomolybdic acid in ethanol (84) and heated for not more than 25-30 minutes. Temperatures greater than 110°C for extended periods of heating caused excessive color development on the background. During heating, blue spots appear on a yellow background (85).

Due to the differences in the  $R_f$  values of taurocholic and glycocholic acids as shown in Figure 1, the spots were sufficiently separated to remove from the plates. Taurocholic with a lower  $R_f$  value of 0.07



Samples from 1-3 are: (1) glycocholic standard, (2) 80 percent ethanolic extract of the small intestine of rats, and (3) taurocholic standard.

Figure 1. Thin-layer chromatogram showing the separation of bile salts.

remained close to the origin; whereas glycocholic with a  $R_f$  value of 0.48 was located near the top of the plate (84). The spots were scraped from the plates and quantitatively transferred to scintillation vials. Preliminary experimentation showed no improvement when samples were eluted from the silica gel with acetone or methanol; thus this step was omitted. Ten milliliters of scintillation liquid made by adding 12 g of Packard PPO (2,5-diphenyloxazole) per liter of toluene were added to each vial. The radioactivity of each sample was counted by the Picker Nuclear Liquimat 220. The glycocholic:taurocholic ratio for each sample was computed by comparing the counts per minute of the taurocholic and glycocholic samples; as follows:

$$\text{cpm} = \frac{8192 \text{ counts}}{\text{time}}$$

The ratio was computed by:

$$\frac{\text{cpm glycocholic}}{\text{cpm taurocholic}}$$

#### STATISTICAL ANALYSIS

The Olivetti-Underwood Programma 101 was used for the statistical analysis of all the data. Data were analyzed by Duncan's new multiple range test (86).

## CHAPTER IV

### RESULTS

The glycocholic:taurocholic ratio was used to indicate differences in the conjugation of taurine with cholic acid. The G:T ratios, their decimal equivalents, and the percentages of increase are shown in Table 3. The percentages of increase were determined by using Diet 3 as a control. A small ratio which converts to a small decimal equivalent indicates relatively large amounts of taurocholic acid formation.

Diet 3 which has been considered the "normal" diet (0.10 percent sulfate with 0.40 percent added cysteine) had the smallest G:T ratio which indicated that the rats fed this diet had the greatest degree of taurine conjugation. Diet 4 which was high in inorganic sulfur (0.42 percent) and with no added cysteine produced the smallest relative amount of taurocholic acid as indicated by the largest ratio. The G:T ratio of animals fed Diet 4 was 272 percent greater than the G:T ratio of those animals fed Diet 3. The G:T ratio of animals fed Diet 1 also differed significantly from those fed Diet 3 ( $P < 0.05$ ). Rats fed Diet 1 exhibited a G:T ratio 214 percent greater than those rats fed Diet 3. Due to large standard deviations, other statistically significant differences were difficult to detect. However, trends can be seen when the G:T ratio and the percentages of increase given in Table 3 are compared to those of Diet 3, the so-called "normal" diet.

TABLE 3

Glycocholic:taurocholic acid ratios of an 80 percent ethanolic extract of the small intestine from rats fed varying levels of inorganic sulfur, organic sulfur, and total sulfur as sulfate<sup>1</sup>

Diet	Level of Sulfur as Sulfate			Glycocholic:taurocholic		Percent Increase
	Inorganic %	Organic %	Total %	Ratio	Decimal Equivalent	
1	0.0002	0.67	0.67	1/19	0.052 $\pm$ 0.029 <sup>a2</sup>	214
2	0.0002	0.57	0.57	1/29	0.034 $\pm$ 0.018 <sup>ab</sup>	106
3	0.10	0.57	0.67	1/61	0.016 $\pm$ 0.004 <sup>b</sup>	0
4	0.42	0.25	0.67	1/16	0.061 $\pm$ 0.030 <sup>a</sup>	272
5	0.42	0.57	0.99	1/28	0.035 $\pm$ 0.015 <sup>ab</sup>	114

<sup>1</sup>Values represent averages of 10 values obtained from determinations of G:T ratios from 5 animals  $\pm$  standard deviation of the mean.

<sup>2</sup>Mean values in a column followed by the same superscript letter do not differ significantly. (P > 0.05).



## CHAPTER V

### DISCUSSION

The significance of the results of this study has strong implications for health. As has been indicated throughout this thesis, bile acid conjugation has been associated with cholesterol metabolism and atherosclerosis. Animals which conjugate the majority of their bile acids with glycine have been found to be more susceptible to hypercholesterolemia and atherosclerosis; whereas taurine conjugation has been associated with a low serum cholesterol level and little atherosclerosis. Therefore, considering the results of the present study, the highest relative conjugation of bile acid with taurine occurred in the experimental group which was fed Diet 3. This diet has been characterized as the "normal" diet and it seems that this diet is most conducive to good health in relation to cholesterol metabolism. In addition, the data from this present study fit the initial hypothesis that taurine conjugation is influenced by the availability of taurine in the tissues. The diet which caused the rats to excrete the least amount of cysteine sulfur as urinary taurine (Diet 4, Table 4) also produced the lowest relative conjugation with taurine. However, by comparing the other data given in Table 4, it is indicated that some other factors must also influence bile acid conjugation. For example, rats fed Diet 1 produced the largest amount of urinary taurine but had the second most limited degree of relative conjugation with taurine; whereas rats fed Diet 3 had the highest degree of relative conjugation with taurine but were limited in urinary

TABLE 4

Comparison of urinary taurine excretion and degree of taurine conjugation of rats fed varying levels of inorganic sulfur, organic sulfur, and total sulfur as sulfate

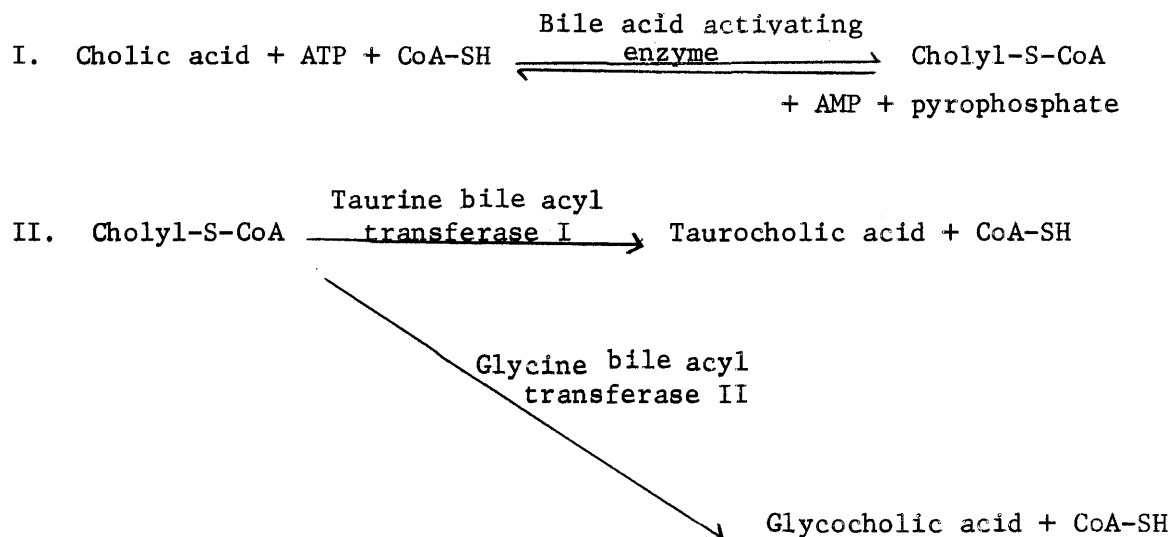
Decreasing Amounts of Urinary Taurine <sup>1</sup>		Decreasing Relative Amounts of Taurocholic Acid	
Diet <sup>2</sup>	cpm x 10 <sup>-3</sup>	Diet	Decimal Equivalent of G:T Ratio
1	3.30	3	0.016 ± 0.004
2	2.92 <sup>3</sup>	2	0.034 ± 0.018
5	1.88 <sup>3</sup>	5	0.035 ± 0.015
3	1.40	1	0.052 ± 0.029
4	0.60	4	0.061 ± 0.030

<sup>1</sup>Whittle, B. A. 1970 The influence of dietary sulfate on the excretion of <sup>35</sup>S-cysteine sulfur as <sup>35</sup>S-aurine sulfur by the rat. Unpublished Ph.D. Dissertation, The University of Tennessee, Knoxville.

<sup>2</sup>Diets 1 and 2 were low in sulfate (0.0002 percent); Diet 1 contained 0.53 percent added cysteine and Diet 2, 0.40 percent added cysteine. Diet 3, so-called "normal" sulfate, (0.10 percent) contained 0.40 percent added cysteine. Diets 4 and 5 were high in sulfate (0.42 percent); Diet 4 contained no added cysteine and Diet 5, 0.40 percent added cysteine.

<sup>3</sup>These figures were normalized to a common value for Diet 3.

taurine excretion. This study was not designed to measure other factors which affect taurine conjugation; however, it seems likely that the differences between the tissue availability of taurine and the relative amounts of taurocholic acid formed would be due to other requirements of conjugation. Bremer (40) stated that the conjugation of cholic acid with either glycine or taurine required the presence of CoA and ATP and was stimulated by magnesium and manganese. The following reaction scheme was given:



Bremer (40) also indicated that conjugation was inhibited by cysteine and cystine in low concentrations and suggested that low concentrations of cysteine and cystine catalyze the oxidation of the sulfhydryl group of CoA.

Rats which were fed Diet 4, which has no cysteine supplement and hence a restricted cysteine supply, had the most limited relative taurine conjugation as well as the lowest urinary taurine levels. Considering the need of CoA for conjugation, it seems that possibly the animal which

was forced to satisfy its sulfate requirement by the oxidation of cysteine sulfur might have a limited amount of cysteine available for the formation of CoA since the oxidation of cysteine sulfur has been shown to be a wasteful process (39). Diet 1 and Diet 2 are similar diets which force the rat to synthesize sulfate except that Diet 1 is supplemented with cysteine. However, as can be seen in Table 4, the relative conjugation of taurine by rats fed these two diets is not identical. Feeding both of these diets resulted in a lower relative taurine conjugation than feeding Diet 3. It is more difficult to explain the difference in relative taurine conjugation by rats fed Diet 1 and Diet 2. But since rats fed Diet 1 have been shown to excrete the most urinary taurine (39), presumably, they would also produce the most pyruvate. Metabolism of the extra pyruvate would put an additional strain on the requirement for CoA. Therefore, if limited CoA is the reason for the decreasing relative amounts of taurine conjugation by rats fed Diet 3 versus Diet 2, then the increased CoA requirement for pyruvate metabolism by rats fed Diet 1 could explain the relative decrease in taurine conjugation.

All the above hypotheses are based on the assumption that the Michaelis constants ( $K_m$ ) for taurine bile acyl transferase I and glycine bile acyl transferase II are different. The Michaelis constants were not found in the literature; however, the lack of rate change by glycine feeding (41) compared with the obvious effects of taurine strengthen this assumption. But, if the Michaelis constants are not different, the effects of CoA and cysteine discussed above would cause the same changes in glycine conjugation as in taurine conjugation resulting in no change in the G:T ratio.

Only the relative position of Diet 5 remains to be explained. Recent data<sup>2</sup> in our laboratory have indicated that high levels of dietary sulfate resulted in the increased storage of the carbon skeleton of cysteine as glycogen. Storage of the carbon skeleton of cysteine as glycogen might tend to limit the amount of taurine available for conjugation.

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<sup>2</sup>Smith, J. T. Unpublished observations.

## CHAPTER VI

### SUMMARY

The relationship of dietary sulfate and the availability of taurine in the tissues to the relative conjugation of bile acids in rats was investigated.

The G:T ratio of animals fed the "normal" diet which contained 0.10 percent inorganic sulfur and 0.57 percent organic sulfur was the smallest indicating the highest relative conjugation with taurine. Increased taurine conjugation in relation to glycine conjugation has been associated with low serum cholesterol levels and limited atherosclerosis. The diet containing a high inorganic sulfur level (0.42 percent) and no added cysteine had the largest G:T ratio corresponding to the smallest relative amount of taurocholic acid. Likewise, in the diets which contained low levels of inorganic sulfur, the G:T ratios were larger than that of the "normal" diet. These data have been interpreted to indicate that either too high or too low a level of inorganic sulfur causes changes in the relative conjugation of taurine. Requirements other than taurine availability which affect bile acid conjugation were also considered in relation to the present findings. The results of this study have been interpreted to imply that the level of inorganic sulfur in the diet may serve an important regulatory function in bile acid conjugation.

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## VITA

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